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Growth Characteristics and Production of Bioactive Compounds in Aromatic Ginger (*Kaempferia galanga*) Callus under Photoperiod and Auxin Treatments

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Abstract

In this study, we investigated the effect of photoperiod and auxin growth regulators (Naphthalene Acetic Acid (NAA) and 2, 4 Dichlorophenoxyacetic Acid (2, 4 D)) on the growth characteristics and synthesis of secondary metabolites of *K. galanga* by callus culture. Growth characteristic variables observed included fresh weight, dry weight, and callus morphology. Spectrophotometry methods measured the observation of total phenolic and flavonoid. Levels of ethyl p-methoxycinnamate and profiles of secondary metabolites in the ethanol extract of callus and rhizomes were observed by Gas chromatography–mass spectrometry (GC-MS). The results showed that photoperiod (P) and auxin (A) treatments affected the growth characteristics and production of callus secondary metabolites. The 16/8 h (light/ dark) of photoperiod treatment combined with 1 mg.L^{-1} auxin 2, 4-D treatment resulted in a maximum fresh weight of 5.52 ± 29 g, not significantly different from 16/ 8 h photoperiod and 1.5 mg.L^{-1} NAA Treatment (P3A5). The best callus dry weight of 0.26 ± 0.05 g was obtained after 16/8 h (light/dark) of photoperiod treatment. The 2, 4-D treatment produced a callus that was friable in creamy-white color, while the auxin NAA produced a greenish and compact callus. Phytochemical study of *K. galanga* callus extract showed the highest accumulation of phenolics and flavonoids in the photoperiod of 16/8 h (light/dark), respectively 0.483 ± 0.065 mg GAE.g^{-1} DW callus and 0.108 ± 0.07 mg QE.g^{-1} DW callus. Ethyl para-methoxycinnamate (EPMC) was formed in almost all treatments. The highest levels of EPMC were formed in the 2 mg.L^{-1} NAA treatment with a 12-h photoperiod of 0.37 mg.g^{-1} DW callus. The secondary metabolites profile in the callus ethanol extract is dominated by aldehydes, saturated hydrocarbons, fatty acids, and their derivatives. It can be concluded that the photoperiod and auxin treatments provided diverse growth variations, with better growth rates, differentiation and production of callus bioactive compounds when using photoperiod 16/8 h (light/dark) and NAA auxin in this study. © 2023 Friends Science Publishers

Keywords: Photoperiod; Auxin; Callus growth; Secondary metabolites; Callus culture of *K. galanga*

Introduction

The aromatic ginger plant (*Kaempferia galanga* L.) is a bioactive chemical source with many benefits. Traditional medicine, phytopharmaca, food and beverage flavoring, spices, and cosmetics use *K. galanga* as a raw material (Narayanaswamy and Ismail 2015). They contain phenolic compounds from the phenylpropanoid group, especially ethyl-cinnamic and ethyl p-methoxycinnamate, which are the main constituents in medicine. *K. galanga* is used in medicine as an anti-inflammatory and analgesic, treatment of headaches, toothaches, rheumatism, anti-tumor and cancer, sedative activity, anti-microbial and anthelmintic (Shetu *et al.* 2018).

The need for natural raw materials such as plant secondary metabolites for the pharmaceutical, food and beverage and cosmetic industries continues to increase. Public awareness of utilizing natural ingredients as medicine and body care impacts the rising demand for quality *K. galanga* rhizomes for raw materials for the pharmaceutical and cosmetic industries. However, the production and quality of *K. galanga* rhizomes produced to meet industrial needs are influenced by the growth and development of plants in the field; this relates to environmental conditions such as soil, nutrition, climate, pests, and diseases. In addition, the growth period for the aromatic ginger plant to produce rhizomes takes approximately 9 to 12 months. The resulting rhizomes

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have different secondary metabolite qualities depending on the environment and cultivation practices.

Culture *in vitro*, especially callus culture, is an alternative that can be used to synthesize secondary metabolites (Kalpana and Anbazhagan 2009). Plant cells have the potential to biochemically produce identical phytochemicals to their parent plant under suitable conditions. However, the balance between primary and secondary metabolic activities is dynamic and is influenced by growth, tissue differentiation, and plant development. The production of secondary metabolites (including alkaloids, terpenoids, steroids and phenolics) can be carried out through *in vitro* culture by manipulating the factors that influence the formation of secondary metabolite products. Several manipulations can be carried out, such as variations in the nutritional composition of growth media, growth regulators, light, temperature, pH and elicitors (Collin 2001). The utilization of growth regulators, especially the auxin class, is widely used to induce the growth and production of secondary metabolites in the callus.

It was reported that eight cell lines could be identified as stable strains by adding 2, 4-dichlorophenoxyacetic acid (2, 4-D) to *K. galanga* callus culture medium, which was used to produce bioactive substances (Kuen *et al.* 2011). The best callus proliferation rate was achieved with auxin 2, 4-D at a concentration of 1 mg.L^{-1} and the resulting callus contained alkaloids, flavonoids, tannins, saponins, steroids, and ethyl para-methoxycinnamate (Shofiyani and Damajanti 2017). The most efficient ZPT combination with a high rate of callus proliferation was 1 mg.L^{-1} BA + 0.1 mg.L^{-1} NAA and the resulting callus synthesized anthraquinone in *G. umbellate*, while 2, 4-dichlorophenoxyacetic acid was the most suitable auxin to induce brittle yellow callus (Hapsari *et al*. 2011; Anjusha and Gangaprasad 2017).

In addition to using appropriate growth regulators in callus production and the production of secondary metabolites, various supporting environmental factors are essential to support callus growth, such as light. Light environmental factors, especially photoperiod, are recognized as essential in cell morphogenesis and the synthesis and accumulation of beneficial secondary metabolites (Tariq *et al.* 2014). Photoperiod is an important environmental factor that regulates plant growth and development and promotes the proliferation of metabolites in many medicinal plants (Liebelt *et al.* 2019). Plant species adapt to photoperiod changes through various physiological modifications, one of which is changing the accumulation of secondary metabolites.

Photoperiod conditioning of *P. vulgaris* suspension cultures with irradiation times of 12/18, 14/16 and 16/14 h light and dark showed higher levels of biomass and secondary metabolites than the control group (16/8 h light and dark) (Fazal *et al.* 2015). The research (Kumar *et al.* 2020) aims to determine the effect of light periods (bright, photoperiod (16:8 h) and complete darkness) on callus growth and photosynthetic pigment content in *B. rubra* callus cultures. The results showed that photoperiod effect callus biomass and secondary metabolite content. According to Castro *et al.* (2019), the optimal lighting environment to promote growth and essential oil content of *Lippia alba* seedlings cultivated *in vitro* is 24-h lighting d^{-1}. Other studies have shown how light affects callus biomass production and accumulation of bioactive metabolites in *Pyrostegia venusta* (Coimbra *et al.* 2017), callus culture of *Verbena officinalis* L. (Kubica *et al.* 2020) and the callus culture of *Olea europaea* L. (Mohammad *et al.* 2019). In the callus culture of *Linum usitatissimum*, the impact of light on the regulation of morphogenetic changes, synthesis of bioactive secondary metabolites and antioxidant capabilities has been widely proven (Zahir *et al.* 2018).

Based on several studies that have been conducted show that the use of callus culture in the development of the production of secondary metabolites has a promising future. This method has shown encouraging results in obtaining raw materials for the pharmaceutical, food, and cosmetic industries by modifying environmental factors that support callus growth during culture. The success of callus culture depends on the biomass and yield of bioactive compounds produced during cultivation. Therefore, it is necessary to do an extensive study about the production of bioactive compounds and growth characteristics in *K. galangal* callus *in vitro* under the influence of photoperiod and the growth regulator auxin.

Materials and Methods

Plant material and culture conditions

Rhizomes of *K. galangal* L. Var. Galesia-2 was obtained from the Bogor Indonesian Drug and Spice Research Institute. Callus induction from rhizome bud and callus subculture was carried out in 30 mL of medium containing 8 g per liter of agar, 3% sucrose with the addition of 2, 4-D and BAP (1 mg.L^{-1} and 0.1 mg.L^{-1}). Calluses were incubated under white fluorescent tubes (TL-D 18 W, Philips Electric®) at 1300 ± 50 lux and 25 ± 2°C for 16/8 h in light and dark. Callus propagation was carried out in the same medium and environment for callus induction. The propagated callus was used as material in this study.

Photoperiod and auxin treatment

An exponential phase callus weighing 0.3 g which had been developed on the multiplication medium, was then planted in 30 mL of media containing 8 g per liter of agar, 3% sucrose, and media pH range 5.6–5.8, with the addition of auxin (2, 4-D and NAA) according to treatment, namely 2, 4-D 1 mg.L^{-1} (A1), 2, 4-D 1.5 mg.L^{-1} (A2), 2, 4-D 2 mg.L^{-1} (A3), NAA 1 mg.L^{-1} (A4), NAA 1.5 mg.L^{-1} (A5) and NAA 2 mg.L^{-1} (A6). The callus was irradiated using a white fluorescent lamp with an exposure time adjusted for the treatment photoperiod (P1). 8/16 h, (P2). 12/12 h and

(P3). 16/8 h dark and light. Callus grown under treatment conditions was used to test growth parameters and callus phytochemical analysis.

Growth parameters

Callus aged six weeks under treatment conditions were then used as samples for observing growth parameters, including callus morphology, callus color, callus fresh weight and dry weight callus. Callus cross sections were observed with a light microscope at 100 and 400X magnification. The photo was taken using an Olympus CX 23 microscope connected to an OM-20 camera.

Preparation of callus phytochemical analysis samples

The callus ethanol extract of K. galanga was prepared using a modified maceration method (Subedi *et al.* 2014). One gram of callus dried at 60°C for 48 h was extracted using absolute ethanol at a ratio of 1:5, sonicated for 30 min and macerated for 24 h at room temperature. The collected filtrate was filtered using a 0.2 mm syringe and stored in dried bottles at 4°C. The extract obtained was then used for phytochemical testing, including determining the total phenolic and flavonoid content and ethyl p-methoxycinnamate.

Determination of total phenolic: The total phenolics of the extracts were determined using the Folin and Ciocalteu reagent, following the method described by Singleton and Rossi (1965) with slight modifications (Chandra *et al.* 2014). The assay was performed in 96-well plates. Sample and standard readings were made using a BioTek Epoch Microplate Spectrophotometer at 765 nm against the reagent blank. The test sample (0.1 mL) was mixed with 0.3 mL of water and 0.1 mL of Folin-Ciocalteu's phenol reagent (1: 1). After 5 min, 0.5 mL of saturated sodium carbonate solution (8% w/v in water) was added to the mixture and the volume was made up to 1.5 mL with distilled water. The reaction was kept in the dark for 30 min and after centrifuging, the absorbance of blue color from different samples was measured at 765 nm. The total phenolic content was calculated as gallic acid equivalents ($GAE.g^{-1}$) of dry callus material based on a standard curve of gallic acid (20–100 mg. L^{-1}, $Y = 0.00109X + 0.1934$, $R^2 = 0.9832$). All determinations were carried out in triplicate.

Determination of total flavonoids: The total flavonoids of the extracts were determined using the aluminum chloride colorimetric method (Chandra *et al.* 2014). For total flavonoid determination, quercetin was used to make the standard calibration curve. Stock quercetin solution was prepared by dissolving 5.0 mg quercetin in 1.0 mL methanol, then the standard solutions of quercetin were prepared by serial dilutions using methanol (5–25 mg. L^{-1}). An amount of 0.6 mL diluted standard quercetin solutions or extracts were separately mixed with 0.6 mL of 2% aluminum chloride. After mixing, the solution was incubated for 60 min at room temperature. The assay was performed in 96-well plates. The absorbance of the reaction mixtures was measured against blank at 420 nm wavelength with a BioTek Epoch Microplate Spectrophotometer. The concentration of total flavonoid content in the test samples was calculated from the calibration plot ($Y = 0.0344X + 0,0915$, $R^2 = 0.9745$) and expressed as mg quercetin equivalent ($QE.g^{-1}$) of dried callus material. All the determinations were carried out in triplicate.

Determination of ethyl p-methoxycinnamate (EPMC) and profiling samples of the ethanol extract *K. galanga* callus: Measurement of ethyl p-methoxycinnamate (EPMC) levels was carried out using gas chromatography-mass spectrometry (GC-MS) analysis using a Shimadzu GCMS-QP 2010 SE series instrument (Shimadzu Corporation, Japan). GC-MS is equipped with an autosampler. Rxi-5 Sil MS column, non-polar fused silica capillary column (30 m length × 0.25 mm diameter × 0.25 µm thickness). GC oven temperature settings: 128°C; pressure: 75.2 KPa; total flow time: 13.1 mL/min; flow rate in column 1.12 mL/min, with the carrier gas being helium. The EPMC content was calculated as the equivalent of EPMC (mg/g dry weight of callus) with a calibration curve made using EPMC standards (Sigma Aldrich) in the concentration range of 0 – 100 mg. L^{-1}, with the results of the equation obtained $y = 14799x – 108130$. The chemical constituents of EPMC were identified by comparison of mass spectra and their retention indices in data in the NIST 08, FFNSC 1.2 and Wiley 8-Mass Spectral libraries of the GCMS data software system. Profiling of essential oils in callus samples was identified with the same tool using Kumar (2014).

Statistical analysis

Version 6.400 of Costat's software program was used to process the data. The ANOVA test examined data with a normal and homogenous distribution. The Kruskal-Wallis analysis is used for data analysis if the data does not match the conditions. The Duncan Multiple Range Test (DMRT) was used in subsequent testing, with a 95% confidence level.

Results

Effect of auxin and photoperiod treatment on callus growth parameters

Fresh weight and dry weight callus: Callus was grown on MS media with a combination of auxin and photoperiod treatment. Callus growth was observed to determine its effect on the callus's fresh and dry weight (Table 1). Photoperiod treatment (P) and auxin (A) significantly affected callus growth parameters. The auxin 2, 4-D 1 $mg.L^{-1}$ treatment combined with the 16/8 h (light/dark) photoperiod treatment gave the highest fresh weight of 5.52 ± 0.29 g, which was not significantly different from the 16/8 h photoperiod combination and NAA 1.5 $mg.L^{-1}$ (P3A5), combined effect

Table 1: Effect of photoperiod (P), auxin treatment (A) and interaction between photoperiod and auxin (P x A) on fresh weight, dry weight and callus morphology of *K. galanga*

Treatment	Fresh Weight (g)	Dry Weight (g)	Callus Texture	Callus Color
PHOTOPERIOD (P)				
8/16 (P1)	3.54 ± 0.86b	0.17± 0.06b	friable, compact	creamy white , greenish
12/12 (P2)	3.75 ± 0.769b	0.20± 0.05b	friable, compact	creamy white, greenish
16/8 (P3)	4.62 ± 0.57a	0.26± 0.05a	friable, compact	creamy white, greenish
AUXIN (A)				
2, 4-D. 1 (A1)	3.88 ± 1.36b	0.18 ± 0.05c	friable	creamy white
2, 4-D. 1.5 (A2)	3.94 ± 0.78b	0.19 ± 0.06bc	friable	creamy white
2, 4-D. 2 (A3)	3.73 ± 0.43b	0.18 ± 0.03c	friable rather compact	creamy white
NAA. 1 (A4)	3.73 ± 0.73b	0.21 ± 0.05bc	compact	creamy white, greenish
NAA. 1.5 (A5)	3.89 ± 0.87b	0.23 ± 0.06b	compact	creamy white, greenish
NAA. 2 (A6)	4.66 ± 0.63a	0.27 ± 0.06a	very compact	greenish
INTERACTION (P x A)				
P1A1	2.76 ± 0.55f	0.13 ± 0.01	very friable	creamy white
P1A2	3.90 ± 0.39cde	0.15 ± 0.01	very friable	creamy white
P1A3	3.36 ± 0.29 def	0.14 ± 0.02	friable	creamy white
P1A4	3.43 ± 1.20def	0.19 ± 0.08	compact	creamy white, greenish
P1A5	3.06 ± 0.24ef	0.20 ± 0.04	compact	creamy white, greenish
P1A6	4.75 ± 0.77abc	0.23 ± 0.05	compact	creamy white, greenish
P2A1	3.37 ± 0.85 def	0.17 ± 0.03	friable	creamy white
P2A2	3.19 ± 0.68ef	0.19 ± 0.07	friable	creamy white
P2A3	3.90 ± 0.48cde	0.18 ± 0.01	friable rather compact	creamy white
P2A4	3.52 ± 0.14def	0.20 ± 0.04	compact	creamy white, greenish
P2A5	3.65 ± 0.22def	0.20 ± 0.04	compact	creamy white, greenish
P2A6	4.85 ± 0.88abc	0.25 ± 0.06	very compact	greenish
P3A1	5.52 ± 0.29a	0.25 ± 0.01	friable	creamy white
P3A2	4.73 ± 0.15abc	0.24 ± 0.02	friable	creamy white
P3A3	3.92 ± 0.34cde	0.21 ± 0.03	friable	creamy white
P3A4	4.25 ± 0.29bcd	0.25 ± 0.03	compact	creamy white, greenish
P3A5	4.95 ± 0.21ab	0.29 ± 0.05	compact	greenish
P3A6	4.38 ± 0.10 bcd	0.33 ± 0.08	very compact	greenish

A = Auxin (2, 4-D and NAA); P = Photoperiod; Mean values following distinct letters under different treatments within a column are significantly different at $P \leq 0.05$ (Duncan's multiple range test); observations were made after 6 weeks of culture

photoperiod 16/8 h and NAA 2 mg.L^{-1} (P2A6), combined effect photoperiod 16/8 h and 2, 4-D 1.5 mg.L^{-1} (P3A2), combined effect photoperiod 8/ 16 h and NAA 2 mg.L^{-1} (P1A6) and the combined photoperiod treatment of 16/8 h and 2, 4-D 1.5 mg.L^{-1} (P3A2) were respectively 4.95 ± 0.21, 4.85 ± 0.88, 4.75 ± 0.77 and 4.73 ± 0.15 g. As a comparison, the treatment combination that gave the lowest callus fresh weight was the combination of 8 h photoperiod and 2, 4-D 1 mg.L^{-1} (P1A1) treatment with a weight of 2.76 ± 0.56 g.

The 16/8 h of photoperiod treatment gave the highest dry weight, 0.26 ± 0.05 g, significantly different from other photoperiod treatments. In contrast, the 8/16 h photoperiod treatment gave the lowest dry weight, which was not significantly different from the 12/12 h photoperiod weighing 0.17 ± 0.06 g and 0.20 ± 0.05 g, respectively. Meanwhile, the auxin treatment with the highest callus dry weight was found in the 2 mg.L^{-1} NAA treatment, 0.27 ± 0.06 g, significantly different from other auxin treatments. The lowest dry weight was found in the 2.4 D treatment 1 mg.L^{-1}, 1.5 mg.L^{-1} and 2 mg.L^{-1}, respectively 0.18 ± 0.05 g, 0.19 ± 0.06 g and 0.18 ± 0.03 g.

Callus morphology: The morphological characteristics of the callus formed showed that the 2, 4-D auxin treatment resulted in a friable and creamy-white callus (Table 1). In contrast, the NAA treatment showed the characteristics of a compact green callus. Callus formation in the NAA treatment led to the process of organogenesis, clearly visible in the callus section (C), where the callus began to undergo a process of cell differentiation leading to the formation of buds (arrows). Callus cells formed in the 2,4-D treatment were undifferentiated (F) (Fig. 1).

Effect of photoperiod and addition of auxin on callus phytochemical analysis of *K. galanga* L.

Total phenolic content (TPC): The 16/8 h of photoperiod treatment gave the highest total phenolic content, namely 0.483 ± 0.065 mg GAE.g^{-1} DW callus, which was not significantly different from the 12/12 h photoperiod treatment, namely 0.462 ± 0.07 mg GAE.g^{-1} DW callus. At the same time, the 8/16 h photoperiod treatment showed the lowest total phenolic content, namely 0.423 ± 0.069 mg GAE.g^{-1} DW callus. These results indicated that the irradiation treatment affected phenol production in the tested *K. galanga* callus (Fig. 2).

Total flavonoids content (TFC): The total flavonoid content of the ethanol extract samples of *K. galanga* callus on irradiation and auxin was calculated as mg QE (Quercetin Equivalent) per gram dry weight of callus. The 16/8 h (light/dark) photoperiod treatment gave the highest total flavonoid content, callus 0.108 ± 0.07 mg QE.g^{-1} DW. Significantly different from the photoperiod treatment at

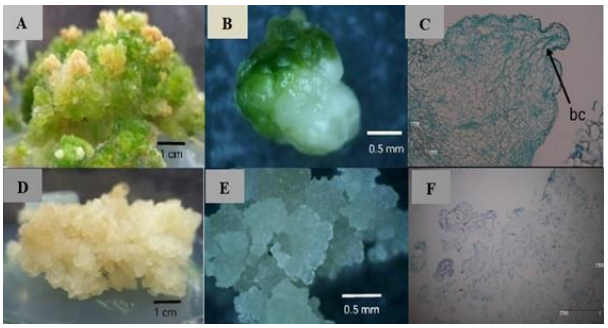

Fig. 1: Callus morphology in NAA (**A**, **B** and **C**) and 2, 4-D (**D**, **E** and **F**) treatment with 16/8 hours of photoperiod (light/dark). Images C and F are cross-sections of callus tissue

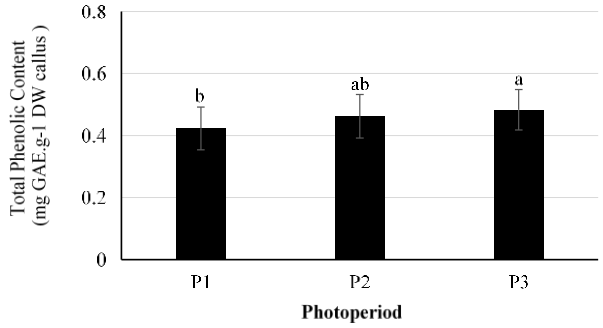

Fig. 2: Graph of the photoperiod treatment effect on total phenolic content (mg GAE.g^{-1} DW callus) in *K. galanga* callus

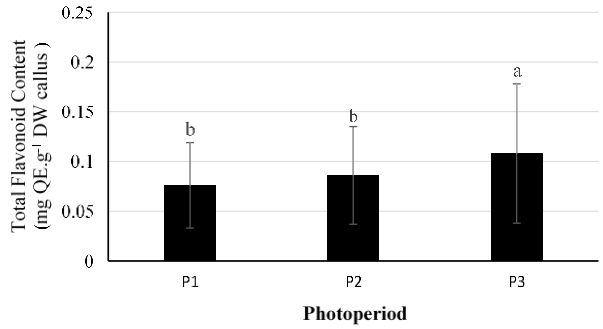

Fig. 3: Graph of the photoperiod treatment effect on total flavonoid content (mg QE.g^{-1} DW callus) in *K. galanga* callus

12/12 h and 8/16 h (light/dark), respectively 0.086 ± 0.049 mg QE.g^{-1} DW callus and 0.076 ± 0.043 mg QE.g^{-1} DW callus (Fig. 3). Meanwhile, the auxin treatment with the highest total flavonoid content was found in the 2 mg.L^{-1} NAA treatment, namely 0.156 ± 0.063 mg QE.g^{-1} DW callus, which was not different from the 1.5 mg.L^{-1} NAA treatment, namely 0.126 ± 0.04 mg QE.g^{-1} DW callus. The auxin treatment that provided the lowest total flavonoid content was found in all 2, 4-D (1–2 mg.L^{-1}) treatments, namely 0.046 ± 0.015, 0.046 ± 0.014, and 0.049 ± 0.013 mg QE.g^{-1} DW callus (Fig. 4). The results of this study indicate that auxin and the level of irradiation given to the callus

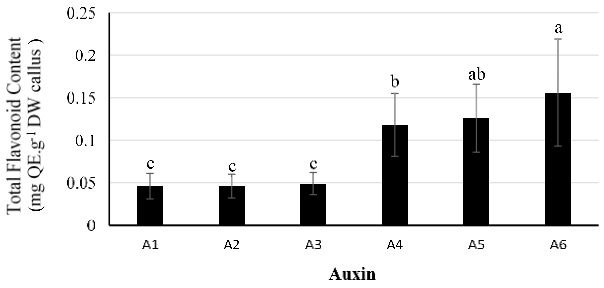

Fig. 4: Graph of the auxin treatment effect on total flavonoid content (mg QE.g^{-1} DW callus) in *K. galanga* callus

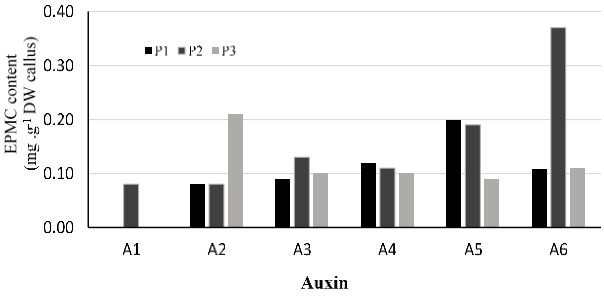

Fig. 5: Graph of the auxin and photoperiod treatment effect on total levels of ethyl para-methoxycinnamic (mg.g^{-1} DW callus) in *K. galanga* callus

during culture affect the production of flavonoids. Exposure to too long light stimulates the accumulation of flavonoids that form in the callus of *K. galanga*.

Determination of ethyl p-methoxycinnamate and profiling samples of the ethanol extract callus: The results of callus testing of the ethanol extract of *K. galanga* with GC-MS treated with photoperiod and auxin showed that EPMC was formed with varying concentrations. EPMC compound was formed in almost all calluses except in the 8 and 16 h of irradiation treatment, combined with auxin 2, 4-D 1 mg.L^{-1}. On the other hand, callus with NAA treatment combined with photoperiod treatment all formed EPMC. The highest levels of EPMC were formed in the 2 mg.L^{-1} NAA treatment with a 12/12 h photoperiod of 0.37 mg.g^{-1} DW callus. Calluses treated with 2, 4-D 1.5 mg.L^{-1} combined with a 16/8 h photoperiod produced an EPMC of 0.21 mg.g^{-1} DW callus.

In contrast, 2, 4-D 1 mg.L^{-1} treatment with photoperiods of 8/16 h and 16/ 8 h did not form EPMS compounds (Fig. 5). The secondary metabolite profile of the ethanol extract of *K. galanga* callus in the 2, 4-D and NAA treatment as determined by GCMS (Table 2 and Fig. 6) shows that the components of the compounds formed are generally saturated hydrocarbons, aldehydes, fatty acids, and fatty acid derivatives. Meanwhile, the ethanol extract of the rhizome was dominated by cinnamic acid, ethyl p-methoxycinnamate, 3-tetradecene, octadecanoic acid, and fatty acid derivatives.

Table 2: The composition of the dominant chemical compound by GC-MS testing on callus *Kaempferia galanga* L in rhizome and callus with auxin (2, 4 D and NAA) treatment

R. time	% Area			Compound	Compound Nature
	2, 4-D	NAA	Rhizome		
6.25	1.28	-		(Z)-1-Chloro-2-(methylsulfonyl)ethylene	Molecules containing Four Carbon Atoms
8.69	-	-	9.17	Ethanol	Organic compund
11.72	-	2.53	-	Eucalyptol	Monoterpenoids
23.9	-	-	0.84	Ethylcinnamate	Sinamic acid ester
24.12	-	-	0.14	1-Dodecene	Hydrocarbons
24.67	-	-	5.95	Dodecane	Aliphatic Hidrocarbon
25.66	3.79	1.32	-	2-methylundecane	Saturated hydrocarbons
27.83	-	1.25	-	D4-methyl glycollate	Esters of glycolic acid
28.13	-	4.12	-	9,12,15-Octadecatrienal	Aldehyde
28.89	-	-	1.13	Ethyl 4-Methoxycinnamate	Sinamic acid
29.029	-	-	0.87	1-Tetradecene	Fatty acids
29.24	-	-	0.95	2,7-Octadien-1-ol, acetate	Acetic acid ester
30.63	-	2.42	-	2, 4, 6-Cyclooctatrien-1-one semicarbazone	Amida
30.66	10.68	-	-	Octadecanal	Aldehyde
30.96	-	-	0.4	Dodecane	Unsaturated hydrocarbons
31.76	-	1.72	-	2-Methylnonane	Saturated hydrocarbons
32.027	2.22	4.59	79.85	Ethyl p-methoxycinnamate	Ester
35.72	-	9.85	-	Octadecanoic acid, methyl ester	Esters of fatty acids
35.74	1.79	-	-	Pentadecanoic acid, 14-methyl-, methyl ester	Esters of fatty acids
36.51	31.35	10.06		Hexadecanoic acid (Palmitic acid)	Fatty acids
37.15	-	5.98	-	Hexadecanoic acid, ethyl ester	Ethyl hexadecanoate
37.15	3.42	-	-	Nonadecanoic acid, ethyl ester	Ethyl nonadecanoate
39.18	-	5.79	-	9,12-Octadecadienoic acid, methyl ester	Esters of fatty acids
39.31	-	16.81	-	9-Hexadecenoic acid, methyl ester	Esters of fatty acids
39.80	-	4.1	-	Octadecanoic acid, methyl ester	Esters of fatty acids
40.02	5.97		-	9,12-Octadecadienal	Aldehyde
40.06	-	4.62	-	9-Octadecenal	Aldehyde
40.13	6.42	-	-	1-Undecene	Alkene
40.49	7.04	3.06	-	Octadecanoic acid, (2-phenyl-1,3-dioxolan-4-yl)methyl ester	Esters of fatty acids
40.60	-	7.26	-	9-Hexadecenoic acid, methyl ester	Esters of fatty acids
41.00	1.65	-	-	Pentadecanoic acid, 4,6,10,14-tetramethyl-, methyl ester	Esters of fatty acids
41.08	-	2.9	-	Octadecanoic acid, ethyl ester	Esters of fatty acids
43.20	-	2.83	-	7-Hexadecenoic acid, methyl ester	Esters of fatty acids

Discussion

Callus culture was developed as an alternative for producing secondary metabolites in plants. The success of callus culture can be determined based on the biomass and the yield of secondary metabolites. The use of growth regulators, nutrient media and growth conditions provided in the right balance determines the success of callus culture. This study investigated the photoperiod factor and the use of auxin-type growth regulators. The results showed that both treatments significantly affected the growth and production of *K. galanga* callus bioactive compounds.

Photoperiod treatment and adding auxin to the callus had various effects on the growth characteristics of the formed callus. There is a tendency to use NAA auxin at a more extended photoperiod (16/8 h slight and dark) to give the best callus growth with a compact and green texture. Photoperiod treatment up to 12/12 and 16/8 h showed an increase in fresh weight (1.06 and 1.3 times, respectively) and dry weight (1.18 and 1.53 times, respectively) compared to the 8/16 h photoperiod treatment (bright /dark). A 2 $mg.L^{-1}$ auxin NAA treatment increased fresh weight and dry weight by 1.25 and 1.5 times, compared to the lowest fresh weight in the 2, 4-D 2 $mg.L^{-1}$ treatment.

Previous research on callus *Linum usitatissimum* showed that light and dark treatment (16/8 h) for four weeks of culture was the optimal treatment to produce the highest fresh and dry weight (Zahir *et al.* 2018). In *Basella rubra* L. callus culture, adding 0.1 $mg.L^{-1}$ NAA and 6 $mg.L^{-1}$ BAP in media at continuous light and photoperiod (16:8 h) supported maximum callus biomass production (Kumar *et al.* 2020). Photoperiod has a significant effect on the increase in fresh callus weight of *Brassica napus* L and *Commiphora wightii* (Arnott) (Tavakkol *et al.* 2011; Kumawat *et al.* 2020), callus morphogenesis of *C. wightii* (Arnott) (Verma *et al.* 2019), best proliferation of *Punica granatum* L callus (Kumar *et al.* 2018) and callus induction of *Cuminum cyminum* leaf explants (Soorni *et al.* 2012).

Observations of callus morphology showed the formation of friable and creamy-white in the 2, 4-D treatment, in contrast to the callus morphology with a greenish color and compact texture formed when using NAA auxin in all photoperiod conditions. The formation of different colors and textures in this study was more due to differences in the use of auxin (Fig. 1). Tavakkol *et al.* (2011) reported that the callus formed on Brassica napus L on media with the addition of NAA and 2, 4-D (2 $mg.L^{-1}$) was greenish and creamy, even in light. The growth of

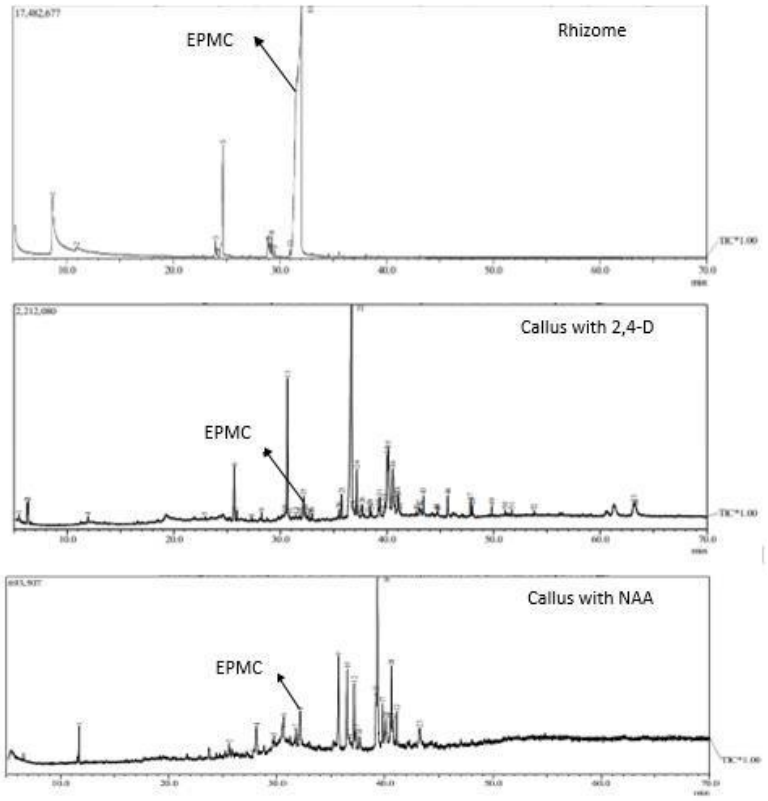

Fig. 6: Profile of secondary metabolite using gas chromatography-mass spectrometry: Profile of secondary metabolite in callus and rhizome *K. galanga*

Gynura procumbens callus from leaf explants supplemented with 2, 4-D produced a brownish-yellow callus (Indriani *et al.* 2017), as well as on the callus *Gynochthodes umbellata* (Anjusha and Gangaprasad 2017). White-brown calluses with a friable texture were found in media added at a concentration of 2, 4-D. (0.5 mg.L^{-1}). In contrast, the callus on MS media reinforced with NAA and IAA was green and had a dense texture (Bano *et al.* 2022). Hesami and Daneshvar (2018) observed that the callus of *Ficus religiosa* was more compact and greenish in the NAA and IBA treatments compared to MS media supplemented with 2, 4-D and to the callus *Basella rubra* which multiplied on NAA and BAP under the light (Kumar *et al.* 2020).

Differences in callus color and texture tend to be due to differences in the type of auxin used (2, 4-D and NAA) in the media in this study. The inhibitory effect of auxin on the formation of chlorophyll causes a difference in the color of the callus formed (reddish and green). Growth regulator 2, 4-D inhibits chlorophyll production better than auxin NAA, so green callus is generally formed on media containing NAA (Tavakkol *et al.* 2011). Siddique *et al.* (2014) observed that using growth regulators of different types and concentrations (separately or in combination) on MS media could change the color and texture of callus. The dynamic characteristics of auxin cause various effects on callus formation and development at different concentrations in culture media (Ren *et al.* 2010).

According to Gaspar *et al.* (2003), using exogenous growth regulators helps the production of endogenous phytohormones, thereby affecting the concentration of internal enzymes and plant hormones. Get another selection by Pasternak *et al.* (2002), callus growth during culture can be caused by the addition of exogenous auxin (2, 4-D and NAA) to the tissue culture medium, which can increase the accumulation of endogenous auxin (IAA) in cells. The increase in auxin concentration causes the Aux/IAA protein to be degraded by the 26S proteasome so that the gene suppression at the start of auxin activation stops; this condition causes auxin to be expressed (Vain *et al.* 2019). The impact of changes in endogenous enzyme and auxin concentrations affected the color, texture and type of callus formed in this study.

Callus growth of *K. galanga* was better with the increased light setting. On the other hand, a lower photoperiod causes a decrease in biomass accumulation in the callus. The availability of sufficient light increases callus morphogenesis and accumulation of callus biomass. Light is an important environmental factor that regulates growth, development, morphogenesis, metabolism, and chlorophyll content in plant cell cultures, tissues, and organs. In addition, light can also affect the effectiveness of growth regulators and the adjustment of endogenous hormone

levels in tissues (Chen *et al.* 2019). This explains that combining the optimum concentration of growth regulators and lighting can increase cell sensitivity to reactivate the cell cycle and activate specific genes for callus proliferation. This can also increase the speed of cell formation, callus mass, and morphogenesis in the *K. galanga* callus in this study.

The callus culture method has been proven to increase the production of secondary metabolites in several types of plants. The production of secondary metabolites *in vitro* culture can be done by optimizing the influencing factors such as media, nutrients, growth regulators, elicitors, precursors and controlled environment. Light is one of the environmental factors that control the physiological processes of cells during the culture period. This study studied the production of bioactive phenolic compounds in *K. galanga* callus culture with auxin treatment and the photoperiod.

The increase in the photoperiod given impacted the levels of total phenolic and total flavonoids callus. Irradiation for 16/8 and 12/12 h of light-dark increased the phenolic content by 1.15 and 1.1 times compared to the 8/16 h of light-dark treatment. The total flavonoid content increased by 1.43 and 1.14 in the light-dark 16/8 and 12/12 h photoperiod treatments compared to the light-dark 8/16 h treatment. The NAA treatment gave better total flavonoids than the auxin 2, 4-D treatment at all concentration levels. The NAA treatment (1, 1.5, and 2 $mg.L^{-1}$) increased the total flavonoids in the callus by 3.4, 2.74, and 2.7 times respectively, compared to the 2, 4-D treatment at the lowest concentration level. The increase in photoperiod and the auxin treatment affected callus's metabolic processes, especially the biosynthesis of phenol compounds. They have increased the quality of the formed *K. galanga* callus, namely increased levels of total phenolic and flavonoids. Phenol compounds are widely proven to function as antioxidants which have benefits for improving human health.

This is in line with the research of Kumar *et al*. (2020); a one-week-old callus grown at 16 h of irradiation had the highest total phenolic content (TPC) compared to another callus at various stages of *B. rubra* callus growth. Fazal *et al.* (2016) found a significant correlation between SOD, POD, and TPC synthesis in cell suspension cultures exposed to various photoperiods. Boron deficiency and nitrogen restriction in Gamborg B5 media combined with light and dark photoperiod treatment for 16 h for four weeks have increased phenolic production, flavonoid and lignan content in *Linum usitatissimum* cultures (Zahir *et al.* 2018). Total phenolics and flavonoids were substantially greater in *Cnidium officinale* callus grown under dichromatic light (red: blue) compared to other regulated light conditions (Adil *et al.* 2019). In many different plant species, such as *Pyrostegia venusta* (Loredo-Carrillo *et al.* 2013), Chinese bayberry (Niu *et al.* 2010) and tomato (Løvdal *et al.* 2010), the effects of light quality and intensity (photoperiod regime) on flavonoid production have been observed to show an increase.

Light plays a vital role in forming and producing secondary metabolites in callus culture. Light is one of the most important ecological factors in controlling the production of plant bioactive compounds and antioxidant activity (Liu *et al.* 2018; Adil *et al.* 2019). It has been reported that light induces chloroplast development leading to the synthesis of precursors involved in various secondary metabolite biosynthetic pathways (including phenols and flavonoids) in *in vitro* culture of plants (Zahir *et al.* 2018). Many medicinal plants, such as *V. officinalis* (Kubica *et al.* 2020) and olive (*Olea europaea*), showed comparable increases in bioactive chemicals in callus cultures under light treatment (Mohammad *et al.* 2019).

Continuous light pressure increases the accumulation of flavonoids (Zahir *et al.* 2018). Stress conditions due to different lighting impact differences in the production of secondary metabolites plant as a form of protection for cells from injury (Fazal *et al.* 2016). As photo protectors and free radical scavengers, flavonols play an essential role in such conditions (Koes *et al.* 2005). In the biochemical pathway, light induces the formation of the A-rings and B-rings of flavonoids, the production of which is accelerated by light. Light controls the first step of using phenylalanine into B-rings modulated by the enzyme phenylalanine ammonia-lyse (PAL) and several other flavonoid-synthesizing enzymes experience increased activity after being treated with light (Salisbury and Ross 1992). Conversely, the low capacity of light received or the absence of light (darkness) during plant growth and development usually results in the inactivation of specific genes and enzymes. The impact reduces the capacity to increase plant biomass and produces secondary metabolites during development (Zhao *et al.* 2001).

EPMC bioactive component is the dominant compound in *K. galanga* essential oil. EPMC is a crucial constituent of rhizome extract, with maintenance and medicinal functions for humans. This study studied the use of *K. galanga* callus culture for EPMC production. With the light environment factor and auxin growth regulator as a treatment, callus can produce EPMC bioactive compounds. However, the production of EPMC in callus ethanol extract was still deficient, ranging from 0.08 – 0.37 $mg.g^{-1}$ DW callus compared to conventionally cultivated rhizome ethanol extract of 7.86 $mg.g^{-1}$ DW rhizome (data not shown in table). The resulting low EPMC content may be due to the callus formed by poorly differentiated cells. Using 2, 4-D and NAA combined with the photoperiod treatment formed a friable and compact (slightly differentiated) callus structure. The formed callus does not yet produce specialized cells or tissue structures in the form of secretory elements or oil glands (idioblast cells), which function for synthesizing and storing essential oils. According to El-Nabarawy *et al.* (2015), secondary compound synthesis and storage sites in plant cells are often located separately, such

as highly specialized structures containing secretory elements and oil glands.

Idioblast cells are storage sites for secondary metabolites, such as essential oils, resins, mucilages, and tannins (Victório *et al.* 2011). Idioblasts formed in aromatic ginger plants are affected by the age of the plant, whereas the plant ages, the number of oil cells increases. An increase in the number of oil cells causes mature rhizomes to contain more essential oils than young ones (Subaryanti *et al.* 2021). El-Nabarawy *et al.* (2015) showed that *in vitro* conditions did not affect gingerol and shogaol production. Variation of nutrient media and plant regulators (2, 4-D combined with BA/Kin), showed no production of gingerols and shogaols in *Zingiber officinale* due to a lack of callus morphological differentiation (Zarate and Yeoman 1994). Petiard *et al.* (1985), concluded that concentrations of plant regulators such as auxin (2, 4-D) promote cell growth, and higher concentrations of this hormone impair the production of secondary metabolites. Fukui *et al.* (1983) studied the effect of growth regulator (2, 4-D) as auxin on red pigment production in *Echium lycopsis* callus cultures and concluded that pigment biosynthesis could be inhibited by 2, 4-D. In contrast to the results of Pise *et al.* (2012), The maximum accumulation rate of shatavarins was found using media containing 2.0 mg.L^{-1} 2, 4-D with *Asparagus racemosus* cell cultures.

The secondary metabolite profile of the ethanol extract of *K. galanga* callus in the 2, 4-D and NAA treatments was determined by the GCMS test (Table 2), indicating that the components of the compounds formed were dominated by aldehydes, saturated hydrocarbons, fatty acids and their derivatives. Meanwhile, the ethanol extract of the rhizome was dominated by cinnamic acid, ethyl p-methoxycinnamate, 3-tetradecene, octadecanoic acid, and fatty acid derivatives. The compounds formed in calluses have various functions in medicine. Palmitic acid has antiandrogenic, antineoplastic, and hypocholesterolemic functions, 5-Alpha reductase inhibitors (Ali *et al.* 2018) and antioxidant potential (Rajalakshmi *et al.* 2016). Linoleic acid is anti-inflammatory, hypocholesterolemia, cancer prevention, hepatoprotection, anti-arthritis, anti-corona (Ali *et al.* 2018), hepatoprotective, antihistamine, hypocholesterolemia (Tyagi and Agarwal 2017). Fatty acids modulate tolerance, responsiveness, and defense induced by biotic factors, thereby regulating responses to biotic and abiotic stresses (Kachroo and Kachroo 2009). Fatty acid esters can play a role in various plant growth processes and human needs. Fatty acid esters can activate steroid hormone receptors in humans and have various functions in eukaryotes (Schmidt *et al.* 1996) as plant growth regulators with activity similar to gibberellic acid growth regulators (Uranga *et al.* 2016). Fatty acid esters are widely used in the cosmetic/cosmetic industry (Miyahara 2019).

Kumar (2020) reported that around 97.19% of the essential oil content of the hexane extract of *K. galanga* rhizome had been extracted and identified. The main bioactive chemicals isolated from the rhizome of *K. galanga* are ethyl p-methoxycinnamate, ethyl cinnamate, kaempferol, kaempferida, kaempsulfonic acid, kaemgalangol A, xylose, sistargamide B and 3-caren-5-one. Ethyl p-methoxycinnamate is the most abundant compound in the rhizome of *K. galanga*; it has antimicrobial, anti-inflammatory and analgesic activity, hypopigmentation, anti-larvicidal and mosquito repellent, antitumor and cancer, anthelmintic activity, antioxidant activity, sedative activity, treatment of headaches, toothaches, rheumatic, antifungal, and antithrombotic.

Conclusion

The photoperiod (P) and auxin (A) treatments significantly affected the growth characteristics and production of bioactive compounds in *K. galanga* callus in this study. The auxin 2, 4-D 1 mg.L^{-1} treatment combined with the 16-h irradiation treatment gave the highest fresh weight of 5.52 ± 0.29 g, not significantly different from the 16/8 h (light/dark) photoperiod combination and 1.5 mg NAA.L^{-1} (P3A5). The 16/8 h of photoperiod treatment gave the best callus dry weight of 0.26 ± 0.05 g. Brittle callus morphology with creamy white callus color in 2, 4-D treatment, while green color and compact callus consistency were formed when using NAA type auxin in all light treatments. Phytochemical observations of *K galanga* callus extract showed the highest total phenolic and flavonoid yields were found at 16/8 h of photoperiod, respectively 0.483 ± 0.065 mg GAE.g^{-1} DW callus and 0.108 ± 0.07 mg QE.g^{-1} DW callus. The profile of secondary metabolites in callus ethanol extract is dominated by aldehydes, saturated hydrocarbons, fatty acids and their derivatives. It can be concluded that the photoperiod and auxin treatments provided diverse growth variations, with better growth rates, differentiation, and production of callus bioactive compounds when using photoperiod 16/8 h (light/dark) and NAA auxin in this study.

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Author Contributions

AS conducts research and does overall paper writing, Sw contributes as a research supervisor and review articles, Sy assists in data analysis and overall article review, AY provides support for literature search and data collection.

Conflicts of Interest

The authors declare no conflict of interest.

Data Availability

Data presented in this study will be available on a fair request to the corresponding author.

Ethics Approval

Not applicable in this paper.

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